

Spectroscopic Behavior and SOD-Like Activity of the Copper (II) Complex of Hippuric Acid

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SUMMARY. The dimeric copper(II) complex of hippuric acid (Hhipp), of stoichiometry $[\text{Cu}_2(\text{hipp})_4(\text{H}_2\text{O})_4] \cdot 2\text{H}_2\text{O}$ has been thoroughly characterized by means of its infrared, Raman and electronic spectra, which could be interpreted on the basis of the known structural data. The superoxide dismutase-like activity of the complex was investigated using the nitrobluetetrazolium/superoxide reduction assay and compared with that obtained for the native $\text{Cu}_2\text{Zn}_2\text{SOD}$ enzyme, tested under the same experimental conditions. The results show a very important SOD-like activity, suggesting that these type of complexes may be interesting for the future search of new SOD-mimetic drugs.

RESUMEN. "Comportamiento Espectroscópico y Actividad Tipo SOD del Complejo de Cobre(II) con Ácido Hipúrico". El complejo dimérico de cobre(II) del ácido hipúrico (Hhipp) de estequiometría $[\text{Cu}_2(\text{hipp})_4(\text{H}_2\text{O})_4] \cdot 2\text{H}_2\text{O}$ fue caracterizado detalladamente por espectroscopia de infrarrojo, Raman y electrónica. Los espectros pudieron ser interpretados en base a la información estructural conocida. La actividad tipo superóxido dismutasa del complejo fue investigada utilizando el ensayo de reducción del colorante nitroazul tetrazolio por superóxido y comparada con la obtenida para la enzima $\text{Cu}_2\text{Zn}_2\text{SOD}$ nativa, ensayada bajo idénticas condiciones experimentales. Los resultados muestran una importante actividad superóxido dismutasa, sugiriendo que este tipo de complejos pueden ser muy interesantes en la búsqueda de nuevas drogas con actividad SOD.

INTRODUCTION

It has been suggested that superoxide and related free radicals may contribute significantly to sustaining chronic inflammation by promoting connective tissue degradation ^{1,2}. In this context, many copper compounds have shown variable degrees of anti-inflammatory activity. This behavior has been related to facilitation of lysyl oxidase activity required for the repair of connective tissue components and either superoxide dismutase (SOD)-mimetic activity of the administered complexes or their facilitation of $\text{Cu}_2\text{Zn}_2\text{SOD}$ synthesis ^{1,3,4}.

During the last years we have investigated a great number of copper complexes, derived from amino acids and small peptides with different pharmacological activities. During these

studies we demonstrated that some of these compounds present an interesting SOD-like activity ⁵⁻⁷. To extend these studies, we have speculated about the possibility of strengthening the SOD-like activity using complexes containing more than one copper center in their structure.

To perform a first study to verify this assumption we have selected the dimeric complex that Cu(II) forms with hippuric acid (N-benzoylglycine, Hhipp). This acid was firstly isolated by Liebig in 1829 from equine urine (this was the origin of his name, from the Greek *hippos*, horse) and is a metabolite of benzoic acid urinary excretion ⁸.

On the other hand, it is well known that carboxylate ligands easily generate binuclear Cu(II) complexes containing carboxylate bridges ⁹⁻¹¹.

KEY WORDS: Cu(II)-hippurate complex, Electronic spectrum, IR spectrum, Raman spectrum, SOD-mimetic activity.

PALABRAS CLAVE: Actividad tipo-SOD, Complejo Cu(II)-hipurato, Espectro IR, Espectro Raman, Espectro electrónico.

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This is also the case with hippuric acid, although this complex presents a number of unusual structural features¹². Therefore, we have synthesized the binuclear complex derived from this acid, performed a detailed investigation of its electronic and vibrational spectra and tested its SOD-like activity.

MATERIAL AND METHODS

All the reagents used for the synthesis of the complex, as well as the solvents, were analytical grade (Merck). Reagents used for the SOD assays were obtained from Sigma.

The complex, of composition $[\text{Cu}_2(\text{hipp})_4(\text{H}_2\text{O})_4] \cdot 2\text{H}_2\text{O}$, was prepared by reaction of the acid (3.00 g) with a slight excess of $\text{Cu}(\text{CO}_3) \cdot \text{Cu}(\text{OH})_2 \cdot \text{H}_2\text{O}$ over the 2:1 ligand-to-metal stoichiometric ratio, suspended in 100 ml of a 30% methanol/water mixture. The suspension was heated at 50 °C, with constant stirring, during four hours. After this time, the non-reacted carbonate was separated by filtration and the limpid blue solution was concentrated to half its volume over a water bath. After cooling, well developed turquoise-blue crystals were collected by filtration, washed with the cold methanol/water mixture and dried in air during a few days. Analytical results: Found: C, 45.35; H, 5.22; N, 5.77. Calculated for $\text{Cu}_2(\text{C}_9\text{H}_8\text{NO}_3)_4 \cdot 6\text{H}_2\text{O}$: C, 45.58; H, 5.06; N, 5.90 %.

The complex is insoluble in water, chloroform, diethyl ether, ethanol, acetone and dichloromethane, slightly soluble in ethyl acetate, and highly soluble in dimethylsulfoxide.

Electronic absorption spectra of the complex dissolved in dimethylsulfoxide were recorded on a Shimadzu, model 204-04550, spectrophotometer, using 10-mm quartz cells. Infrared spectra were recorded in the spectral range between 4000 and 400 cm^{-1} with a Bruker IFS 66 FTIR instrument, using the KBr pellet technique. Raman spectra were obtained with a FRA 106 Raman accessory of the same instrument. The 1064 nm line of a solid state Nd : YAG laser was used for excitation.

SOD-like activity was investigated by the method of Beauchamp and Fridovich¹³ as improved by Imanari *et al.*^{14,15}. This method is based on the inhibitory effect of SOD over the reduction of nitrobluetetrazolium (NBT) by the superoxide anion generated by the system xanthine/xanthine oxidase, at pH = 12, measuring the absorption changes at 560 nm. The used solvent was dimethylsulfoxide. For comparative purposes we have also measured the activity of

native superoxide dismutase from bovine erythrocytes (Sigma), under the same experimental conditions.

RESULTS AND DISCUSSION

Structure of the complex

This compound presents an interesting and relatively unusual structure. It exists as a bridged dimer with the bridging occurring only through two single carboxyl oxygens. Each Cu(II) cation is five coordinated, having a distorted [4+1] tetragonal-pyramidal geometry. As shown by the schematic drawing of the structure, presented in Fig. 1, each monomeric unit contains a tetragonally arranged copper ion bonded *trans* to the oxygen atoms of two water molecules and to one carboxyl O-atom of two hippurate anions. Angles within this O_4Cu plane are close to 90°. The copper dimer consists of two of these monomeric units bridged through carboxyl oxygens of two hippurates. In each case, one of these O-bridges constitutes the apical ligand to the Cu(II) cation. Thus, each copper (II) ion is bonded to two bridged hippurates (Cu-O distances of 2.37 and 1.91 Å), one uniquely attached hippurate (Cu-O distance of 1.91 Å) and two water molecules (Cu-O distances of 2.00 Å)^{12,16}.

The Cu-Cu separation of 3.33 Å is long enough to preclude any type of metal/metal interaction, a fact which is also supported by the magnetic moment of the complex (ca. 1.9 BM per Cu(II) ion at room temperature) that shows a normal paramagnetic behavior¹².

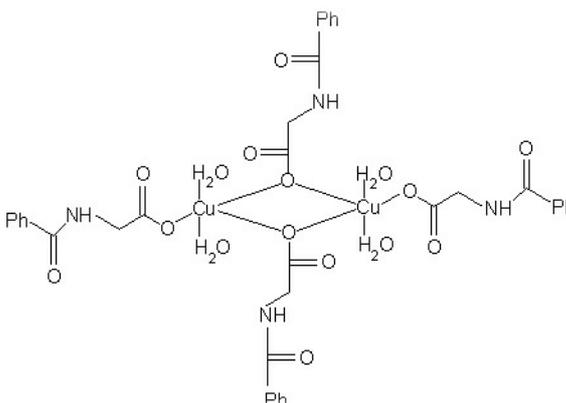


Figure 1. Schematic structure of the investigated $[\text{Cu}_2(\text{hipp})_4(\text{H}_2\text{O})_4] \cdot 2\text{H}_2\text{O}$ complex.

Electronic spectrum

The electronic absorption spectrum, measured in a dimethylsulfoxide solution, presents a relatively broad band centered at 750 nm ($\epsilon = 106 \text{ L} \cdot \text{M}^{-1} \cdot \text{cm}^{-1}$) which is rather asymmetric, sug-

gesting the presence of an unresolved low-energy component, at about 850-860 nm. These two transitions lie in the region which is characteristic for a square pyramidal CuO_5 environment¹⁷, and are originated in two typical “d-d” transitions of the metal center. The relatively high value of the molar absorptivity ϵ , is in agreement with the distorted geometry around this center.

In the higher energy range, the spectrum shows a second, very strong and well defined, absorption band centered at 287 nm ($\epsilon = ca. 4000 \text{ L.M}^{-1}.\text{cm}^{-1}$). This band may be assigned to a $\pi \rightarrow \pi^*$ transition of the phenyl group of hippuric acid¹⁸, although also the presence of a $\text{O} \rightarrow \text{Cu}$ charge transfer in this region cannot be ruled out (*cf.* for example¹⁹).

Vibrational spectra

On the basis of the known structural data, it is possible to attempt to make an approximate assignment of the IR and Raman spectra of the complex to have an additional tool for its rapid characterization (Table 1). The assignment is based on some general literature data²⁰⁻²³ and is briefly commented above.

In the higher frequency region, the IR spectrum is dominated by a relatively broad and strong band, with a defined peak at 3317 cm^{-1} , related to the O-H stretching vibrations of the

two types of water molecules (coordinated H_2O and hydration H_2O) present in the structure. The N-H stretching vibration of the amide moiety is only seen as a shoulder at *ca.* 3430 cm^{-1} , on the higher energy side of this broad band and no Raman counterpart could be found.

A number of bands related to the C-H stretching of both the phenyl ring and the CH_2 group of the ligand could also be assigned in the high energy region. Some bending modes of these moieties, as well as some characteristic groups of ring vibrations, $\nu(\text{CC})$ modes and the $\rho(\text{CH}_2)$ rocking mode have also been identified in other spectral ranges.

The most interesting part of the spectrum, which involves the vibrations of different carbonyl groups is relatively complicated to assign because this spectral range is relatively complex in both spectra, as can be seen from Figure 2.

In this region a total number of three different $\nu(\text{C}=\text{O})$ stretching vibrations is expected: those of the four amide groups (amide I band), those of the two carboxylic groups involved in direct Cu-O bonds and those of the two carboxylic groups related to the bridges. For the amide I band one expects a vibrational band in the range $1680\text{-}1630 \text{ cm}^{-1}$ ²⁰ and for the carboxylic groups acting as monodentate ligands to the Cu(II) center this band is expected at about

Infrared	Raman	Assignment
3430 (?)sh		$\nu(\text{NH})$
3317 vs	3300 w	$\nu(\text{OH})$ water
3025w, 3020w, 3030w	3067 s, 3050sh	$\nu(\text{CH})$ phenyl
2933 w	2943 m, 2871 w	$\nu(\text{CH}_2)$
1628 vs, 1600 sh	1638 m, 1603 vs	$\nu(\text{C}=\text{O})$, $\delta(\text{H}_2\text{O})$
1578 vs	1588 sh	$\nu(\text{C}=\text{O})$
1550 m	1551 m	$\delta(\text{NH})$
1491 m	1490 m	ring modes
1441 m	1440 w	$\delta(\text{CH}_2)$
1417 w, 1402 s	1418sh, 1391 m	ring modes
1327 m, 1315 sh	1329 s, 1310 w	$\nu(\text{C}-\text{O})$
1294 s		$\nu(\text{C}-\text{O})$
1252 w, 1165 w	1250 w, 1152 m	$\nu(\text{CC})$
1078 m		$\delta(\text{CH}_2)$
	1036 w, 1032 m	$\delta(\text{C}-\text{H})$ phenyl in plane
1001 m	999 vs	$\delta(\text{C}-\text{H})$ phenyl out of plane
928 w	936 m	$\nu(\text{CC})$
843 w, 802 w	835 m	ring modes
731 sh	730 w	$\rho(\text{CH}_2)$
692 s	690 w	$\rho(\text{H}_2\text{O})$ (?)
615 m	620 m	$\delta(\text{O}=\text{C}-\text{N})$
555 w	556 w	$\nu(\text{Cu}-\text{O})$ (?)
	513 m	$\nu(\text{Cu}-\text{O})$ (?)

Table 1. Vibrational spectrum of $[\text{Cu}_2(\text{hipp})_4(\text{H}_2\text{O})_4] \cdot 2\text{H}_2\text{O}$ in the spectral range between 4000 and 500 cm^{-1} ; vs: very strong; s: strong; m: medium; w: weak; sh: shoulder.

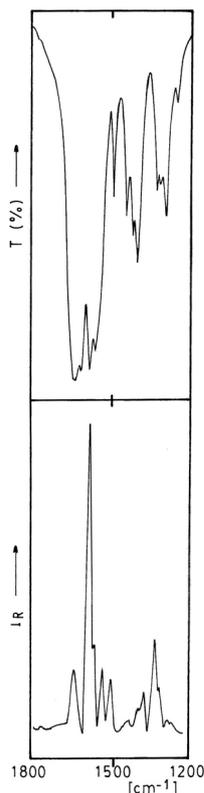


Figure 2. Infrared (above) and Raman (below) spectra of $[\text{Cu}_2(\text{hipp})_4(\text{H}_2\text{O})_4] \cdot 2\text{H}_2\text{O}$, in the spectral range between 1800 and 1200 cm^{-1} .

1630-1600 cm^{-1} ²³. Besides, in the case of the carboxylate groups involved in bridging, this band should be slightly displaced to higher frequencies than in the previous case. This means that all these $\nu(\text{C}=\text{O})$ stretching modes are expected to lie very close to each other and they are not clearly differentiable. On the other hand, in this same region, also the bending mode of the water molecules is expected ²³, additionally complicating this spectral range.

Moreover, the stretching vibrations of the two types of C-O moieties attached to the metal center are no easy to differentiate. In the case of unidentate bonds this vibration is expected to lie between 1315-1370 cm^{-1} ²³ and it should be displaced to somewhat lower frequencies in the bridge bonds.

In the lower frequency region we have tentatively assigned one of the rocking modes of the bonded water molecules and two $\nu(\text{Cu}-\text{O})$ ligand-to-metal vibrations, and could also identify one of the bending modes of the amide moiety (amide IV band) ²⁰.

SOD-like activity

In Table 2 we present the results of the SOD-assay performed at different complex concentrations. In this Table we show the percentage of inhibition of NBT reduction at any con-

Concentration (M)	%-Inhibition
5.00×10^{-5}	92.52
3.33×10^{-5}	91.56
1.66×10^{-5}	77.87
0.83×10^{-5}	67.83
4.165×10^{-6}	63.69
2.5×10^{-6}	52.23
0.835×10^{-6}	45.86

Table 2. Results of the SOD assays.

centration. The 100% of superoxide activity corresponds to an assay performed in the absence of complex. In order to determine the concentration of the complex required to yield 50% inhibition of the reaction, we plotted the percentage of inhibition against the logarithm of metal concentration as shown in Figure 3 and from this plot the I_{50} value can be easily determined. The obtained value was 1.58×10^{-6} M, which compared with the I_{50} value of the native enzyme (9.5×10^{-9} M) gives a relation $[\text{complex}]/[\text{enzyme}] = 166$, which indicates a relatively important SOD-like activity of the investigated complex.

It has been proposed that only complexes with I_{50} values below 20×10^{-6} M may become clinically interesting ². Therefore, the investigated complex clearly fulfills this requirement and appears as an interesting possibility for further investigations in the field of SOD-mimetic drugs.

In this sense, $[\text{Cu}_2(\text{hipp})_4(\text{H}_2\text{O})_4] \cdot 2\text{H}_2\text{O}$ seems to be clearly superior than all the other complexes that we have so far investigated. In the case of Cu(II) complexes of amino acids the I_{50} values are found in the range between 3.0×10^{-5} and 3.7×10^{-5} M ⁵, whereas in most of the

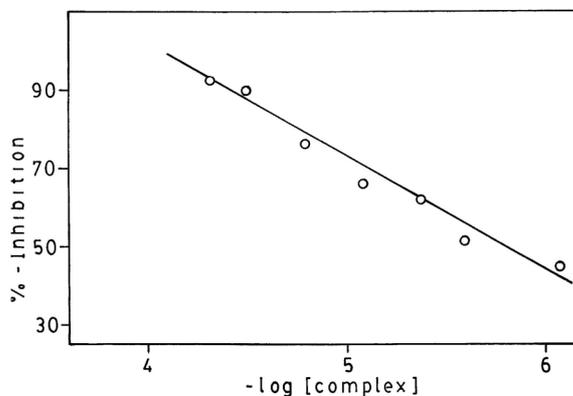


Figure 3. Percentage of inhibition of NBT reduction as a function of the logarithm of $[\text{Cu}_2(\text{hipp})_4(\text{H}_2\text{O})_4] \cdot 2\text{H}_2\text{O}$ concentration.

complexes with simple dipeptides they lie between 9.9×10^{-5} and 2.4×10^{-4} M⁷.

It has been suggested that SOD-like activity is enhanced in the case of complexes with distorted environments²⁴. If the structural peculiarities of $[\text{Cu}_2(\text{hipp})_4(\text{H}_2\text{O})_4] \cdot 2\text{H}_2\text{O}$ are examined in detail^{12,16} it becomes evident that, apart of the [4+1] CuO_5 environment of each Cu(II) center, each of these centers presents a relatively open coordination site on the basis of the pyramid, probably favoring a rapid interaction and exchange of the O_2^- substrate and this interaction can evidently occur on the two metal sites present in the complex, enhancing the activity.

CONCLUSIONS

The IR, Raman and electronic spectra of the dimeric copper (II) complex of hippuric acid of composition $[\text{Cu}_2(\text{hipp})_4(\text{H}_2\text{O})_4] \cdot 2\text{H}_2\text{O}$ could be recorded and adequately interpreted on the basis of its peculiar structural characteristics. The complex also shows an important superoxide-dismutase-like activity suggesting that this type of dimeric complexes may constitute a new and interesting basis for the future search of new and more potent SOD-mimetic drugs.

Acknowledgements. This work has been supported by UNLP (Project X-339) and CONICET. E.J.B. is a member of the Research Career from CONICET.

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